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PRINCIPAL INVESTIGATOR: Gregory Hannon

CONTRACTING ORGANIZATION: University of Cambridge, UK
Cambridge, CB 0RE

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14. ABSTRACT The purpose of this collaborative project is to establish a molecular definition of the dormant state of a cancer cell. In doing this, we hope to understand how this dormancy is broken, ultimately leading to recurrence in a patient that was stably in remission. Once our understanding of this is more complete, it is hoped that we can devise strategies for secondary prevention. This funding year we have pursued an alternative strategy for the identification of dormant tumor cells and the characterization of their microenvironment: Imaging mass cytometry or IMC. Over the past year, we have constructed two antibody panels, which can be read by IMC, one to identify and characterize the status of breast tumor cells and one to characterize immune infiltrates. We plan to apply this approach as an adjunct to STPT, LCM, and RNAseq. We have also aimed to identify candidate dormancy regulators and find ways to manipulate these for patient benefit. Additionally, we have identified asparagine bioavailability as a major regulator of EMT, which could be manipulated to influence response to therapy, affecting the potential pool of residual/dormant disease, and its recognition by the innate and adaptive immune system.					
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1. INTRODUCTION

The primary purpose of our collaborative research project is to discover the genes and pathways that regulate breast cancer dormancy. This information will allow us to generate hypotheses about the mechanisms underlying dormancy maintenance, as well as how it is broken. We will use models of breast cancer wherein murine or human breast cancer cells establish and progress through dormancy. We will utilize innovative methods to perform laser capture microdissection of cancer cells in the dormant state as well as cancer cells that are progressing from dormancy. These cells, which are at distinct stages of cancer progression with their native environments, will be molecularly profiled. We will also profile various cell-types within the micro-environment that contact cancer cells. This molecular description of the dormant state will allow us to perform loss-of-function and gain-of-function studies of candidate drivers and suppressors of dormancy progression. Ultimately, we anticipate the identification of genes that could serve as attractive targets of therapeutic inhibition.

While the Tavazoie lab has been working towards human breast cancer profiling, the Hannon lab has been optimizing conditions for identifying and profiling dormant cells. In the last four years of funding we have been working towards two goals. One is the ability to locate dormant cells and the second is the ability to capture and profile, using RNA seq, single cells.

2. KEYWORDS

Breast cancer, Dormancy, metastasis, EMT, RNA seq, heterogeneity, ASNS

3. ACCOMPLISHMENTS

Task 1 – Broadly to identify dormant tumour cells and characterize these and their environment

We continue to pursue means to identify dormant cells using the strategies outlined in the last progress report. We have been hampered by the extended period of time that it has taken the University of Cambridge to complete the purchase of the STPT. This should now finally arrive in January. This will be complete with the collection device that was mentioned in the prior report. This is capable of collecting 25 micron slices from our selected tissues and automatically transferring these to microscope slides with a >95% capture rate.

In the interim we have pursued an alternative strategy for the identification of dormant tumor cells and the characterization of their microenvironment: Imaging mass cytometry or IMC. This technology allows samples to be stained with up to 50 different antibodies (more conventionally 38). These are read by mass spectrometry using metal mass labels conjugated to the antibodies with extremely high signal to noise ratios and with roughly 1 micron X-Y resolution in situ. Over the past year, we have constructed two antibody panels, one to identify and characterize the status of breast tumor cells and one to characterize immune infiltrates. All but three antibodies comprising these panels have now been validated. By cutting 2 micron sections for use in the instrument, the same cells can be read by both panels and the information integrated.

We are beginning to use this on our own dormancy models, but the most important area that this opens is the use of human tissue samples. We have begun a collaboration with Alana Welm and Cyrus Ghajar who have access to tissues from dormant cites to validate our ability to detect dormant cells and to study their microenvironment. We plan to apply this approach as an adjunct to STPT, LCM, and RNAseq, which we will continue to pursue diligently once the instrument arrives.

Tasks 2 and 3 – to identify candidate dormancy regulators and find ways to manipulate these for patient benefit.

Figure 1

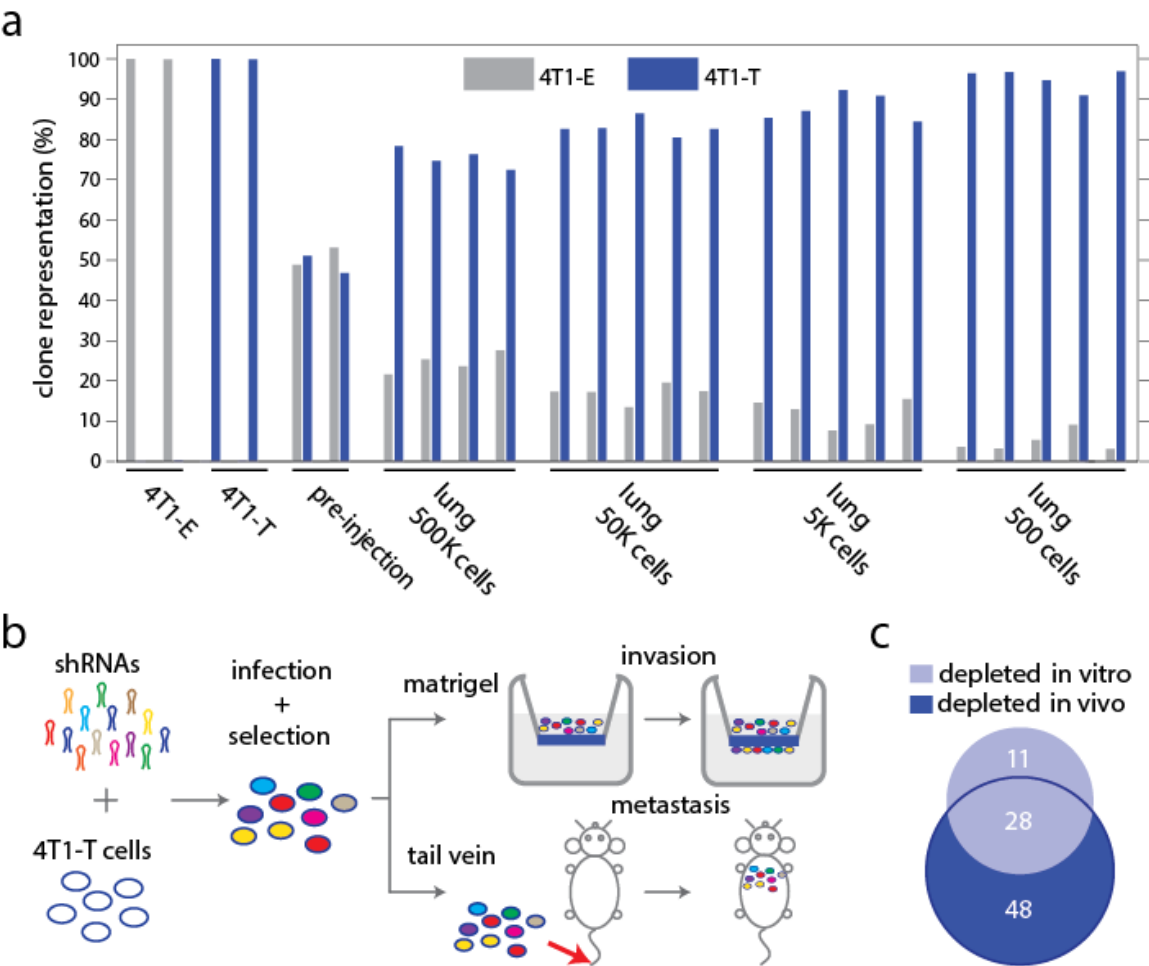


Figure 2

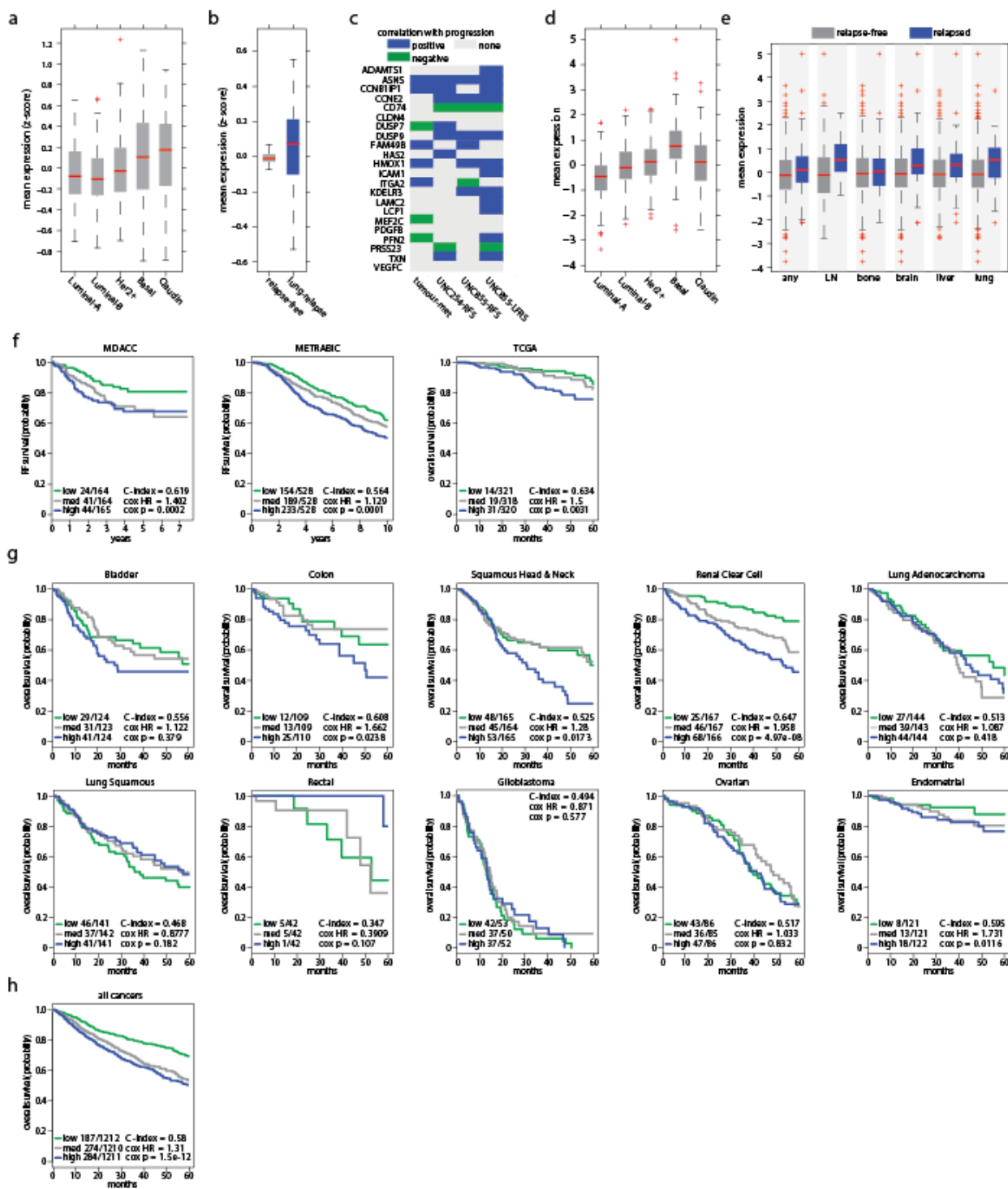


Figure 3

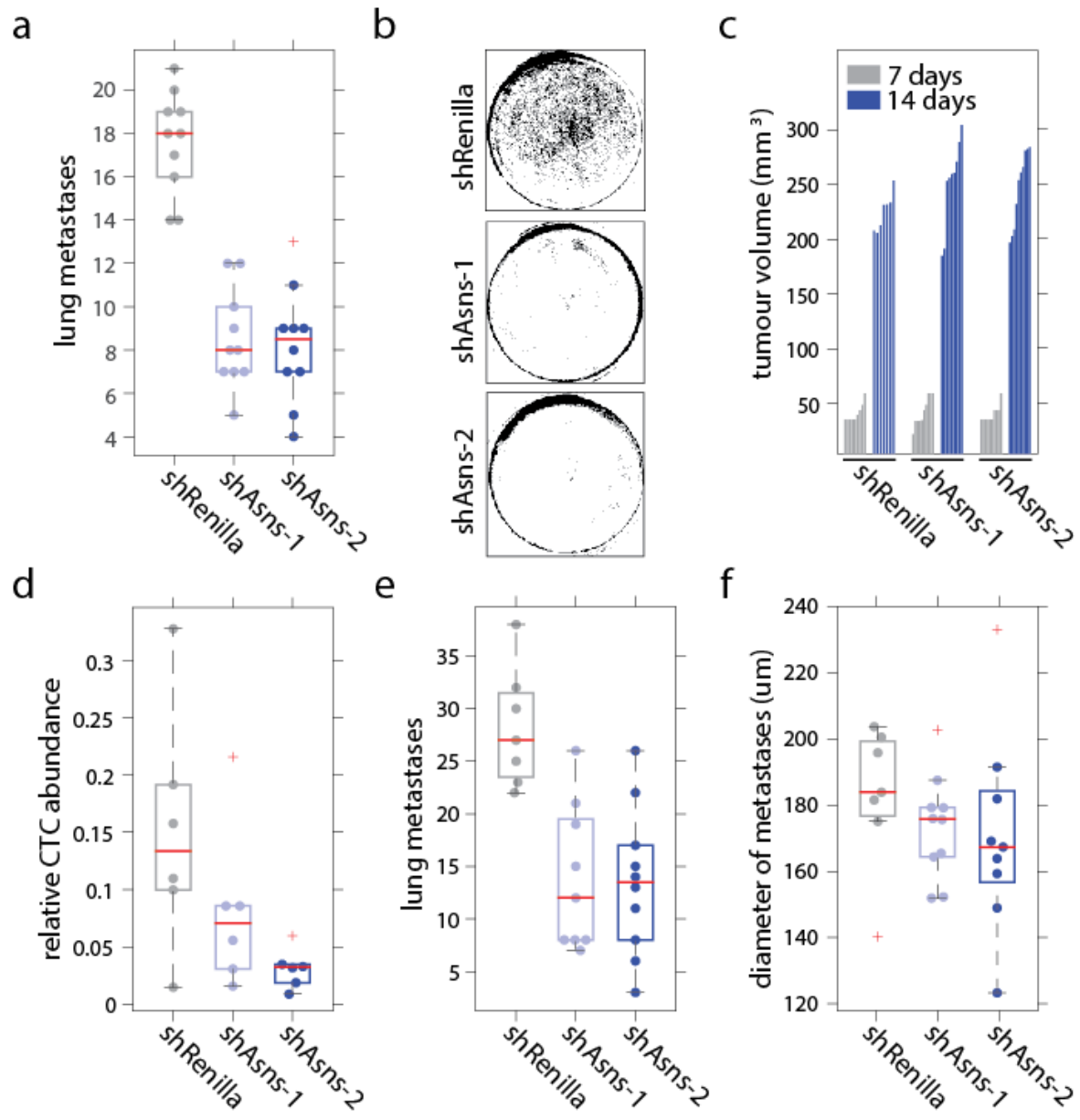


Figure 4

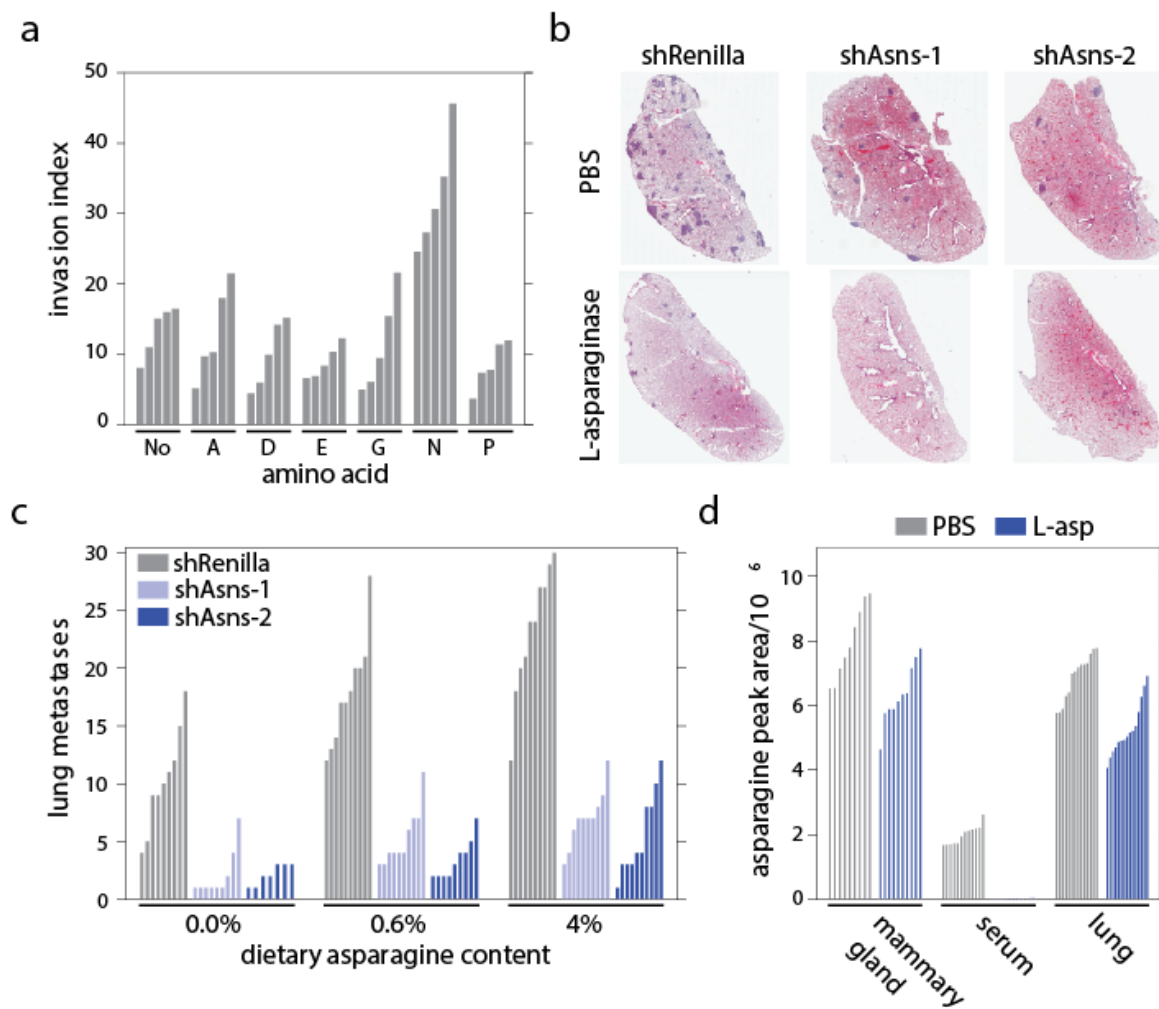
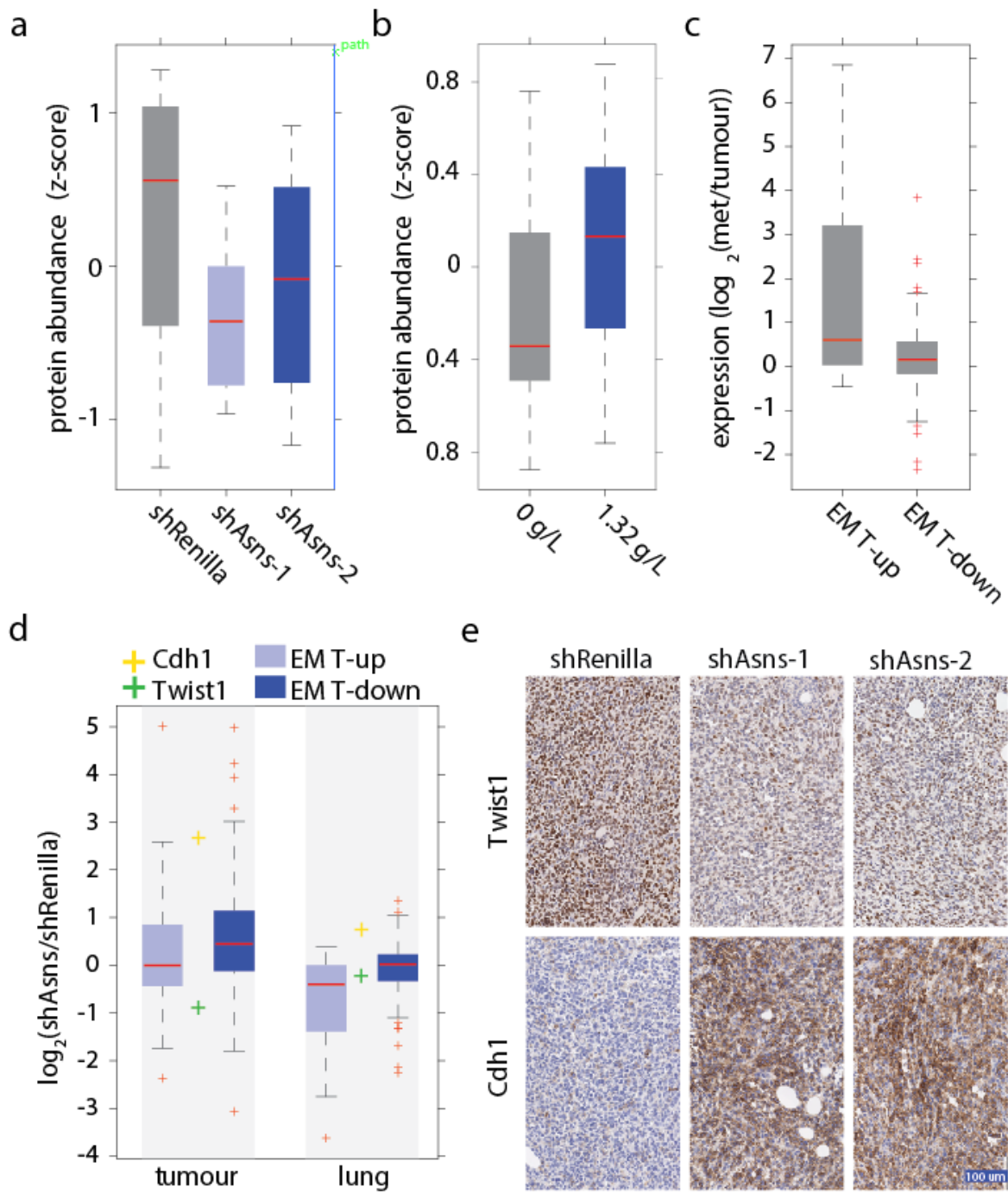


Figure 5



As we awaited the necessary instrumentation to isolate dormant tumour cells, we decided to pursue the question from another angle. Our prior studies of a model of breast tumour heterogeneity revealed a process called vascular mimicry as a driver of metastasis. Only a small fraction of the clones that formed the primary tumour became detectable in the blood, and those that did universally shared the ability to undergo what we are now calling the EET (epithelial to endothelial transition) (Wagenblast et al., Nature 2015). However not all cells that were capable of EET could form secondary metastases. These clones (E and T) did not appear to differ in their ability to survive in the bloodstream, since tail vein injection resulted in similar numbers of each in CTC populations. However, they did differ profoundly in their ability to colonize secondary sites.

To validate the observation that 4T1-T had greater metastatic potential among CTC-proficient clones, we combined equal numbers of 4T1-E and -T cells and introduced these directly into the bloodstream of immune compromised recipients (NOD-SCID-*Il2rg*^{-/-} (NSG) mice). Though the two clones were initially present in equal abundance (Fig. 1a), when cells were harvested from the lung at day 7, clone T predominated, with its relative representation being inversely correlated with the total number of cells injected.

We identified 192 genes with higher expression in 4T1-T than 4T1-E cells. Their corresponding Gene Ontology terms were enriched for processes important for metastatic spread (e.g. cell migration and locomotion). A retrospective analysis of patient data showed that genes within the set are more highly expressed in aggressive breast tumour subtypes. They were also more highly expressed in the primary tumours of patients with later relapse to the bone, brain, and lungs as compared to primary tumours of relapse-free survivors.

To identify metastatic drivers, we carried out an RNAi screen, with two arms (Fig. 1b). In total, 26 pools of ~50 shRNAs, targeting the 192 genes, were introduced into 4T1-T cells. These were placed onto matrigel or introduced into NSG mice by tail vein injection. After 24 hours, the cells that had invaded through matrigel were collected and, after 7 days, lungs were harvested from the mice. Using high-throughput sequencing, we identified shRNAs that were depleted from the invaded cell populations or lung metastases, presumably because they targeted genes important for these processes. Strong overlap was observed when the *in vitro* and *in vivo* candidates were compared (Fig. 1c).

Of the 28 candidate genes that scored in both the *in vitro* and *in vivo* assays, Asparagine Synthetase (Asns) had the most robust clinical evidence supporting its relevance to disease progression. Expression levels of the human orthologue, ASNS, were predictive of general and lung-specific relapse in two breast cancer patient datasets. Also, when a small collection of matched tumour and lung metastases were transcriptionally profiled, ASNS was found to be more highly expressed in secondary lesions. ASNS is more highly expressed in aggressive tumour subtypes and it is more highly expressed in patients with relapse to the lymph node, brain, liver, and lungs as compared to relapse-free survivors. Subsequent analyses identified ASNS as predictive of survival in three additional breast cancer patient datasets (MDACC, METABRIC, and TCGA). In addition to

breast, ASNS is negatively correlated with survival in 4 out of the 10 other solid tumours in the TCGA Pan-Cancer dataset and is a globally predictive biomarker for solid tumours (see Figure 2).

To validate Asns as a metastatic driver, we infected 4T1-T cells with two shRNAs targeting Asns or a control and introduced these cells intravenously into NSG mice. Asns-silenced cells produced significantly fewer lung metastases (Fig. 3a). Asns-silenced cells also showed poor invasion into matrigel (Fig. 3b). Silencing Asns did impact proliferation *in vitro*; however, this defect was minor compared to that observed in the invasion assay. Intracellular free asparagine was reduced by silencing of Asns in 4T1-T cells, and the abilities to invade and proliferate were increased in Asns-silenced cells when media was supplemented with asparagine.

When Asns-silenced cells were injected into the mammary fat pad, no significant change in primary tumour formation was observed (Fig. 3c), yet CTCs and lung metastases were reduced (Fig. 3d, e). Although statistically insignificant, metastases initiated by silenced cells were noticeably smaller, hinting at a growth defect at the metastatic site (Fig. 3f). Similar results were obtained with Asns-silenced parental 4T1 cells, indicating that Asns dependency is not a peculiarity of a single clonal line. Enforced Asns expression in parental 4T1 populations did not affect primary tumour growth but did increase metastases both in number and size. Similar outcomes were observed upon enforced ASNS expression in human MDA-MB-231 breast cancer cells.

To determine whether the observed effects were unique to asparagine, we supplemented media separately with other non-essential amino acids lacking in the culture media, or with glycine, which is present and acted as a negative control, and assayed cells for invasiveness. 4T1 cells responded uniquely to asparagine supplementation, with an approximately 2-fold increase in invasiveness (Fig. 4a), though levels of uptake were similar for each of the amino acids with the exception of aspartic and glutamic acid. More profound impacts were observed with MDA-MB-231 cells. Growth was not affected by asparagine supplementation for either cell line during the same period.

Since invasiveness could be modulated either by altering asparagine biosynthetic capacity or by modifying extracellular pools, we asked whether metastasis could be influenced by treatment with L-asparaginase. This enzyme is used to treat acute lymphoblastic leukaemia (ALL), which is generally highly dependent on extracellular asparagine. L-asparaginase has proven ineffective for treating solid tumours, in accord with Asns silencing not impacting growth at the primary site. NSG mice harbouring orthotopic 4T1 tumours were treated with 60 U L-asparaginase 5 times per week for 19 days, reducing serum asparagine to undetectable levels (Supplementary Table 5). While no significant difference, compared to controls, was detected in primary tumours, a reduction in metastasis was observed (Extended Data Fig. 5a-c).

The availability of extracellular asparagine can also be manipulated by altering asparagine levels in the diet. shRNA-infected 4T1-T cells were orthotopically injected into mice that received either a control, low-

asparagine, or high-asparagine chow (0.6%, 0%, and 4%, respectively). HPLC confirmed that serum asparagine levels were significantly altered in concordance with dietary intake. Asparagine restriction did not impact primary tumour growth, regardless of *Asns*-expression status. In contrast, metastatic burden was decreased in animals that were fed low-asparagine diets and increased in animals given high-asparagine diets (Fig. 4c). Metastases were nearly undetectable in mice that were injected with *Asns*-silenced cells and fed a low-asparagine diet. Similar results were obtained when parental 4T1 cells were orthotopically injected into animals fed these same asparagine-controlled diets.

Analyses of the mammary gland, serum, and lungs of mice by mass spectrometry suggest that, under normal physiological conditions, asparagine levels are highest in mammary gland and lowest in serum (Fig. 4d). High asparagine availability in the mammary gland might blunt the impact of *Asns* silencing or changes in global asparagine levels on primary tumour growth, while low levels in the serum may make CTCs susceptible to these manipulations. Overall, asparagine abundance in tissues correlated with *Asns* expression. *ASNS* expression levels follow a similar pattern across human tissues, raising the possibility of similar impacts if asparagine levels were altered in patients.

To understand the mechanism by which asparagine availability might impact invasion and metastasis, we examined expression changes induced by *Asns* silencing, both at the RNA and protein level. RNA measurements were the strongest predictor of protein-level changes. In accord with a previous report of translational pausing at asparagine residues in L-asparaginase treated cells, we also found asparagine content to be predictive of corresponding protein-level changes, irrespective of whether they were normalized for RNA levels.

Amongst the asparagine-enriched proteins that were depleted upon *Asns* silencing, we found genes whose human orthologues were up-regulated after induction of the epithelial-to-mesenchymal transition (EMT) (Fig. 5a). Overall, depleted proteins had an 18% higher asparagine content than the analysed proteome as a whole, while EMT-up proteins had 20% asparagine content. Human EMT-up proteins are also asparagine-enriched. A reanalysis of existing ribosomal profiling data revealed high rates of pausing at asparagine residues within EMT-up genes in L-asparaginase treated human prostate cancer cells, and these same proteins increased in expression when 4T1 cells were cultured in elevated asparagine (Fig. 5b). Asparagine enrichment is a globally conserved property of EMT-up proteins, with enrichment being greatest in mammals.

EMT-up genes were also down-regulated at the transcriptional level in *Asns*-silenced cells. EMT-up genes were also increased in their mRNA levels when parental 4T1 cells were grown in asparagine supplemented media. A reanalysis of existing data also showed reduced expression of EMT-up genes when ATF4, which regulates *ASNS* transcription, was deleted in haploid cells, and liver cells from L-asparaginase treated ATF4 knockout mice were more perturbed in their EMT program than were similarly treated WT mice. Considered

together, these data suggested that asparagine bioavailability might impact metastasis, at least in part, through regulation of EMT.

To examine the role of EMT in metastasis in our model, we orthotopically injected 4T1-T cells in which we had silenced expression of Tgf- β , a key driver of EMT. Primary tumour growth was unaffected by this manipulation; however the expression of two prototypical EMT markers (Twist1 and E-cadherin) were altered to indicate a perturbed EMT program. Tgf- β -silenced cells produced fewer metastases from the primary tumour or when intravenously injected.

Although no differences were detected in H&E stained tumour sections, morphological distinctions were noticeable when shRNA infected 4T1-T cells were isolated from primary tumours by 6-TG selection, with most Asns-silenced cells displaying an epithelial morphology. The majority of 6-TG isolated metastatic cells displayed a mesenchymal morphology, regardless of Asns-expression status, and this was matched by an increase in the expression of EMT-up genes (Fig. 5c). Nevertheless, EMT-up genes were down-regulated in Asns-silenced vs. –expressing metastatic cells, indicating an increased representation of epithelial cells in the silenced populations. Similarly, EMT-down genes were up-regulated, and Twist and E-cadherin expression measurements indicated a higher epithelial representation in Asns-silenced primary tumour cell populations (Fig. 5d). These results were validated by qPCR for Twist and E-cadherin in Asns-silenced and –expressing cells that were FACS isolated from primary and secondary lesions.

Staining for Twist and E-cadherin proteins confirmed that EMT is perturbed in Asns-silenced tumors, and this same pattern was observed in the corresponding metastases (Fig. 5e). Similar patterns were observed in the primary tumours of mice that had been treated with L-asparaginase or subjected to dietary asparagine restriction.

Our model of breast tumour heterogeneity has strongly implicated asparagine bioavailability as a regulator of metastatic progression. This is also likely relevant in human cancers, as high ASNS expression is a marker of poor prognosis for many tumour types. One mechanism underlying our findings is likely a link between asparagine bioavailability and EMT, which can be observed *in vitro* and *in vivo*.

A vast literature has associated EMT with resistance to therapeutic intervention. This encompasses cytotoxic chemotherapies as well as targeted therapies. Recent studies have even suggested that cells that have undergone EMT can evade immunotherapy. For a cell to remain dormant and serve as a seed of later disease it must evade a patient's initial treatment. The work described above has led us to the hypothesis that asparagine bioavailability, either biosynthetic capacity in the tumour cell itself or the extracellular availability in the microenvironment influences the potential of cells colonizing secondary sites. In high asparagine conditions, cells can adopt a mesenchymal phenotype and potentially occupy the perivascular niches that have been previously proposed as a site for DTC preservation. Restriction of asparagine through any of a number of

mechanisms may negate this potential in the colonizing cell but might also reverse EMT later at the dormant niche and in micrometastases, revealing these cells to the immune system or to therapeutic intervention. Thus, we feel that this work represents a substantive step forward toward the overall goals of this grant.

4. IMPACT

What opportunities for training and professional development has the project provided?

This work has supported the training of a number of individuals. Several technicians have been trained as a part of this program, one of whom has started her graduate studies. Clare Rebbeck has been promoted to Senior Research Associate during her time as part of this award, and a new postdoc Ashley Nicholls is acquiring new skills in drug discovery as we search for an ASNS inhibitor.

How were the reports disseminated to communities of interest?

A manuscript (Knott et al.) has been accepted for publication in Nature

What do you plan to do during the next reporting period to accomplish the goals?

For the remaining term of the grant, we have a number of ambitious goals. We wish to bring to fruition the idea of using the STPT to identify and profile dormant tumour cells, generating candidate that can be taken into precisely the types of studies cited in section 4. We hope to also characterize by IMC the microenvironments, with respect to immune cells, of dormant tumour cells, metastases, and micromets in several tissues from mouse and human. Finally, we hope to pursue our hypothesis that manipulating asparagine bioavailability would at very least improve initial therapy to reduce the pool of cells available to adopt the dormant state and at best, make DTCs vulnerable to either endogenous immune attack or therapeutic intervention.

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

The observation that asparagine availability impacts metastasis could provide strategies for improving primary therapy and for reducing relapse.

5. CHANGES/PROBLEMS

The problems we have had acquiring the necessary equipment have impacted the trajectory of our work in this grant. This did, however, provide the impetus to attempt our alternative approach that has provided very interesting and potentially important new directions.

6. PRODUCTS

Knott et al., Nature, 2017, in press

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project (reporting period 10/01/16 – 9/30/17)?

Name: Clare Rebbeck

Project Role: Senior Research Associate (Post Doc)

Nearest person month worked: 2 months

Contribution to project: Project design, animal work, cell culture & imaging

Name: Ashley Nicholls

Project Role: Research Associate (Post Doc)

Nearest person month worked: 12 months

Contribution to project: Project design, animal work, cell culture & imaging

Name: Aarthi Gopinathan

Project Role: Research Associate (Post Doc)

Nearest person month worked: 7 months

Contribution to project: Animal work, cell culture & imaging

Name: Natasha Narayanan

Project Role: Student

Nearest person month worked: 1 month

Contribution to project: no change

Has there been a change in the active other support of the PD/PS(s) or senior/key personnel since the last reporting period?

The following previously active grants have closed:

- 5 P01 CA013106-44
- 17-A723 STARR

For complete details, please refer to Other Support documentation in the Appendix.

What other organizations were involved as partners?

N/A

8. APPENDICES

Greg Hannon Other Support

OTHER SUPPORT

HANNON, GREG

New York Genome Center

ACTIVE

R37 GM062534-16 (Hannon)

9/15/00 – 8/31/18

0.6 calendar

NIH/NCI

Roles of small RNAs in guarding germ cell genomes

The major goal of this project is: To understand the mechanistic basis of dsRNA-induced gene silencing using a biochemical approach.

Role: Primary Investigator

OVERLAP

None

Cold Spring Harbor Laboratory

ACTIVE

63180501 (Hannon)

01/01/16 – 9/30/19

0.00 calendar* PEW

Charitable Trust

Glaucoma Research

The major goal of this project is: to carry out research to understand further the biology of retinal ganglion loss.

Role: Primary Investigator

OVERLAP

None

Cancer Research UK Cambridge Institute

ACTIVE

W81XWH-12-1-0300 (Hannon)

9/15/12 – 5/14/18

1.2 calendar

Rockefeller Univ PI Tavazoie

DOD Collaborative Scholars Award

Understanding tumor dormancy as a means for secondary prevention

The major goal of this project is: to characterize the molecular and cellular basis of breast cancer dormancy and dormancy progression.

Role: Co-Primary Investigator

5 U01 MH106035-01 (Hannon) NIH	9/26/14 – 6/30/17	1.2 calendar
An Optogenetic Toolkit for the interrogation and control of a single cell The major goal of this project is: to develop a broad optogenetic toolkit, based on covalent protein tags and photoreleasable compounds, enabling the recovery of genetic material, the alteration of gene expression, and the insertion of transgenes to any cell of the brain with high spatial precision. Role: Primary Investigator		
W81XWH-14-1-0110 (Hannon) DOD Breakthrough Award	9/30/14 – 9/29/19	0.6 calendar
The major goal of this project is: to molecularly characterize DCIS Role: Primary Investigator		
0028354 (Hannon) PEW Charitable Trust	4/1/15 – 3/31/20	1.2 calendar
The major goal of this project is: to develop state of the art RNAi tools Role: Primary Investigator		
110161/Z/15/Z (Hannon) Wellcome Trust Investigator Award	8/1/16 – 7/31/23	1.2 calendar
This is scholarship support for work on non-coding RNAs, including piRNAs and lncRNAs. Role: Primary Investigator		
C9545/A24042 CRUK Grand Challenge Award – IMAXT	5/1/17 – 4/30/23	1.2 calendar
The major goal of this project is: to image and molecularly annotate xenografts and tumors. Role: Lead Co-Investigator		
C14303/A17197 Core Award	10/1/14 – 10/1/19	0.6 calendar CRUK
The major goals of this project are: to understand the biological functions and mechanisms of non-coding RNAs; to attempt to tackle critical problems in cancer biology, with a major focus on breast and pancreatic cancer, to understand aspects of disease ranging from the genetics of early, non-invasive cancers to critical pathways driving metastasis; to develop new technologies in several areas including mining information inherent in tumor heterogeneity, optimization of CRISPR-based strategies, and building general platforms for control of biological processes by light . Role: Primary Investigator		

OVERLAP
None

*Please note that no designated level of professional support is required by the funding organization for these projects, however, Dr Hannon has 4.2 calendar months of effort currently available at his discretion to apply to the oversight of these research endeavors and any other research or administrative responsibilities. At no time will his effort exceed 12 person months in any one year.